

Supporting Information

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**Quantitative Analysis of the Fate of Gold Nanocages In Vitro and In Vivo after Uptake by U87-MG Tumor Cells\*\***

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## Supporting Information

### Experimental Section

*Synthesis of AuNCs:* The AuNCs were prepared using a galvanic replacement reaction between Ag nanocubes and HAuCl<sub>4</sub> in water. The Ag nanocubes were prepared by reducing silver trifluoroacetate (Sigma-Aldrich, St. Louis, MO) with ethylene glycol in the presence of poly(vinyl pyrrolidone) ( $M_n \approx 55,000$ , Sigma-Aldrich), and a small amount of sulfide.<sup>[S1]</sup> The samples were harvested by centrifugation and then washed with acetone once to remove the remaining salt precursor and ethylene glycol, and finally washed with deionized water four times. The AuNCs were prepared by adding 0.2 mM HAuCl<sub>4</sub> aqueous solution drop wise into a suspension of the Ag nanocubes dispersed in 1 mg/mL poly(vinyl pyrrolidone) aqueous solution under refluxing.<sup>[30]</sup> After synthesis, the AuNCs were purified by centrifugation at 10,000 rpm and washing with deionized water four times. The nanocages were finally dispersed in deionized water.

*Preparation of the c(RGDyK)-labeled AuNCs:* The c(RGDyK)-labeled AuNCs were prepared by first mixing 1 mL of aqueous dispersion of the as-prepared AuNCs with 1 mL of 1 mM aqueous solution of *O*-pyridyl disulfide-poly(ethylene glycol)-succinimyl valeric acid-activated ester ( $M_n \approx 5000$ , Laysan Bio, Arab, AL). After the reaction at room temperature for 8 h, the AuNCs were purified by repeated centrifugation and washing with deionized water two times, and then they were re-dispersed in 1 mL of phosphate buffered saline (PBS, pH 7.4) containing c(RGDyK), which was purchased from Anaspec Inc. (Fremont, CA). The concentration of the peptide in PBS was 0.5 mg/mL. After reacting at 7 °C for 12 h, the dispersion was centrifuged and the resulting c(RGDyK)-labeled AuNCs were re-dispersed in fresh PBS. The dispersion was stored at 4 °C until future use.

*Cell culture:* U87-MG human brain tumor cells (American Type Culture Collection, Manassas, VA) were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), and 1% antibiotics (containing penicillin and streptomycin, Invitrogen). The medium was changed every other day, and the culture was maintained at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

*Determination of the number of c(RGDyK)-labeled AuNCs per U87-MG cell by UV-vis spectroscopy:* The U87-MG cells were used for uptake studies once they had reached 85–90% confluence. Typically, the cells were incubated with 2 mL of the culture medium containing the

c(RGDyK)-labeled AuNCs at 37 °C in the wells of a 6-well culture plate. After 24 h, the medium was removed from each well, and 1 mL of fresh medium (without AuNCs) was added to wash off the loosely bound AuNCs on the surface of the cells. After repeating the washing procedure one more time, a UV-vis spectrophotometer (CARY50, Varian, Marietta, GA) was used to record extinction spectrum of the culture medium containing the AuNCs. We followed the same experimental procedures described in our previous works.<sup>[32,33]</sup> Briefly, the background from 300 nm to 1100 nm was obtained for EMEM containing FBS and antibiotics. Before uptake studies, we recorded a spectrum of the culture medium containing FBS, antibiotics and the c(RGDyK)-labeled AuNCs (denoted “before”). After incubation of the nanocages with the cells, a spectrum was recorded again for the medium combined with the solutions from the two washing steps (denoted “after”). When the culture medium after uptake was combined with the two washing solutions, the concentration of nanoparticles was reduced as a result of dilution. Therefore, for the latter case, we adjusted the spectrum by accounting for the total volume (2 mL) of the added washing solutions. See Figure S1b for the spectra. We calculated the number of c(RGDyK)-labeled AuNCs per U87-MG cell, based on the extinction spectra and a calibration curve (Fig. S1a), and the number of cells in each well as counted using a hemocytometer.

*Two-photon microscopy (TPM):* TPM was used to quantitatively measure the number of the c(RGDyK)-labeled AuNCs in U87-MG cells *in vitro* as a function of time. The two-photon laser scanning confocal microscope was based on a Zeiss LSM 510 Meta NLO system with a Coherent Chameleon Ti:Sapphire laser, coupled to an upright Zeiss Axioskop 2 microscope. U87-MG cells containing AuNCs were seeded at a very low density of approximately 20 cells in a culture plate. The photoluminescence intensity of each mother cell at day 0 was recorded, and the position of each cell was marked. The culture medium was changed immediately after imaging and the plate was placed back into the incubator for culture. The intensities of the daughter cells were recorded again at day 2 and correlated with their corresponding mother cells within the range of the marked position. It should be noted that each pair of daughter cells came from a single mother cell because the cells were initially seeded very sparsely and the migration of the cells during the 2-day culture period should not be significantly enough to get to the proximity of other cells. The laser power at the two time points did not vary more than 1%.

*Animal handling and photoacoustic microscopy (PAM):* All *in vivo* animal experiments were carried out in compliance with the Washington University Institutional Animal Care and Use

Committee. Athymic nude mice weighing about 25 g (5–6 weeks) were used for the *in vivo* experiments. The mouse was anesthetized with a mixture of ketamine (85 mg/kg) and xylazine (15 mg/kg). After the uptake studies, U87-MG cells bearing the c(RGDyK)-conjugated AuNCs were harvested by trypsin treatment, and 20  $\mu$ L of the cell suspension ( $1 \times 10^7$  cells/mL) was subcutaneously injected into the dorsal side of the mouse. During PAM imaging, we maintained the anesthesia of the mouse by using vaporized isoflurane (1 L/min oxygen and 0.75% isoflurane, Euthanex Corp., Allentown, PA), and vitals were monitored using a pulse oximeter (8600 V, Nonin Medical INC., Plymouth, MN). The body temperature of the mouse was maintained by using a water-heated pad. Immediately after imaging, the mouse was brought back to a mouse cage. The PAM imaging was repeated with the same procedure every 2 days for 6 days. After the last imaging session, the mouse was euthanized by administering an overdose of pentobarbital.

The details about PAM were well described elsewhere.<sup>[15, S2]</sup> Photoacoustic waves were excited by a pulsed laser at 770 nm and detected by a single-element ultrasound transducer (5, 10, or 50 MHz center frequency). The dark-field light illumination was coaxially and confocally aligned with the ultrasound focus. By measuring photoacoustic signals based on the time of arrival, one-dimensional depth-resolved images, referred to as A-lines, were acquired. Scanning along a transverse direction enabled the construction of two-dimensional depth-resolved images (B-scans), and further scanning along the other traverse direction provided three-dimensional images of heterogeneous optical absorptions in a sample. The acquired three-dimensional raw data could be presented in two forms: a maximum amplitude projection, which is the projection of the maximum photoacoustic amplitude along each A-line onto the orthogonal plane; or a three-dimensional image rendered by Volview software (Kitware, Clifton Park, NY). The axial and transverse resolutions were primarily determined by the ultrasonic transducers, which were 15 and 45  $\mu$ m for the 50 MHz transducer, 125 and 140  $\mu$ m for the 10 MHz transducer, and 150 and 560  $\mu$ m for the 5 MHz transducer, respectively.<sup>[34, S2]</sup> The maximum penetration depths at the ultrasonic frequency of 50 MHz and 5 MHz were *ca.* 3 mm and *ca.* 30 mm in biological tissues, respectively.<sup>[S2, S3]</sup>

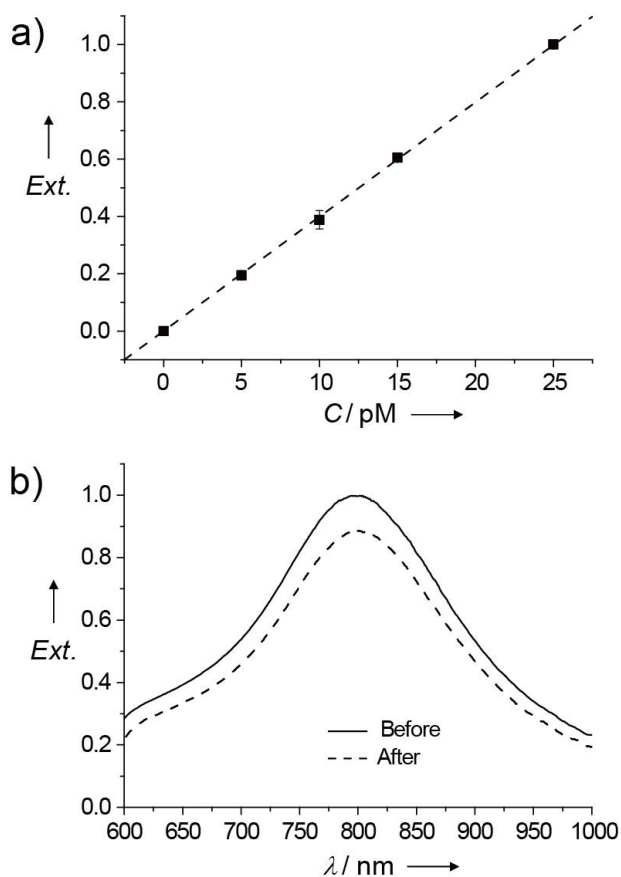
To obtain the *in vivo* calibration curve for PAM imaging of U87-MG tumors, 20  $\mu$ L of the U87-MG cell suspensions ( $1 \times 10^7$  cells/mL) containing different numbers of AuNCs per cell were injected subcutaneously in parallel into the dorsal side of the same mouse. PAM images were then acquired immediately after the injection and the photoacoustic amplitudes were plotted as a function of the number of AuNCs.

*Determination of cell numbers of U87-MG tumors:* In parallel control groups, at 2, 4, and 6 days post-injection of U87-MG cells in the nude mice, the tumors (n=3) were excised and dissociated into single cell suspensions by repeated steps of collagenase and trypsin digestion. The number of cells at each time point was counted using a hemocytometer.

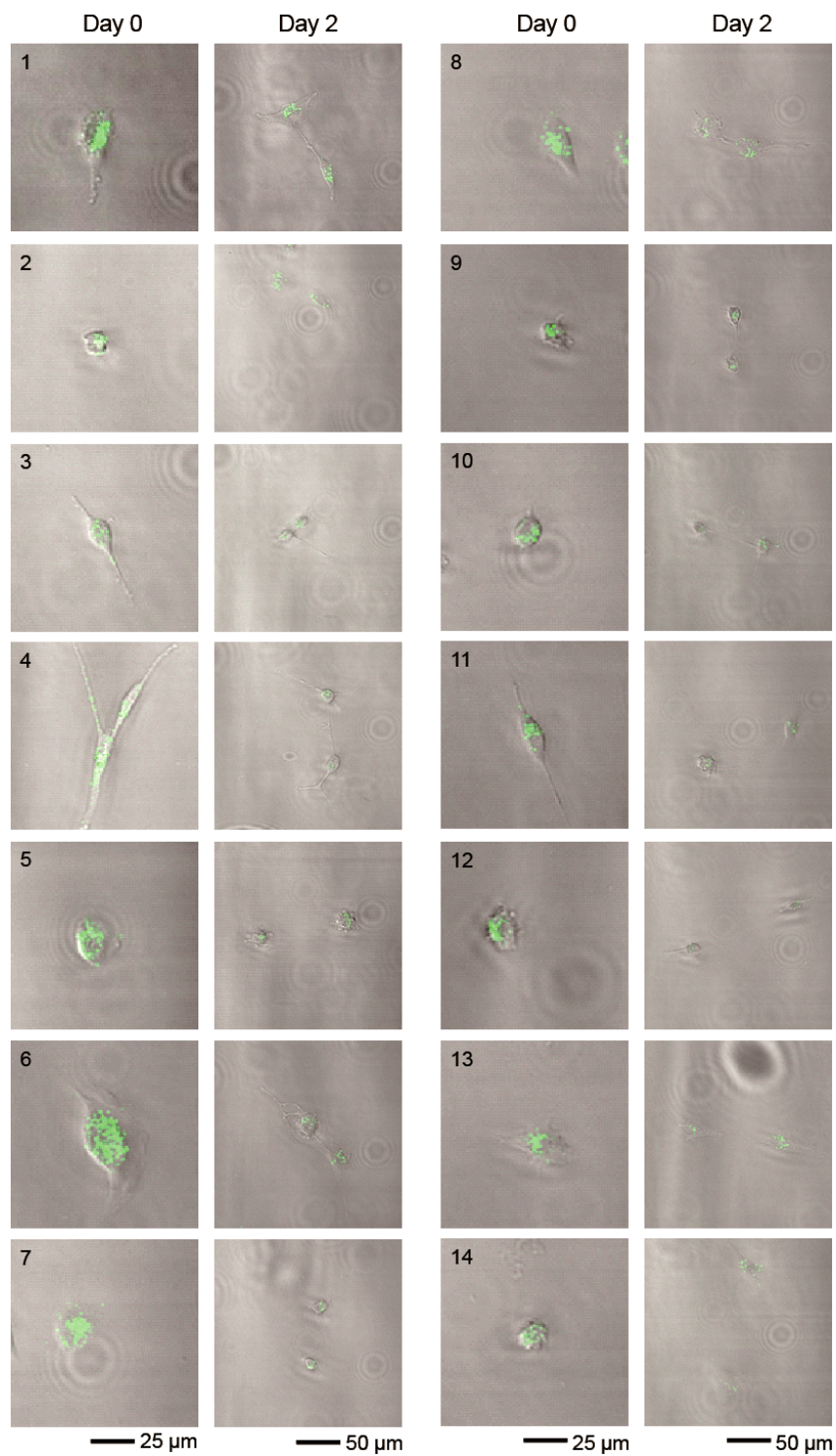
*Statistical analysis:* All the data plots were summarized as means  $\pm$  standard deviations. The number of AuNCs per cell for the two daughter cells (Fig. 2d), as obtained from two photon microscopy, were compared using analysis of variance (ANOVA).

## References

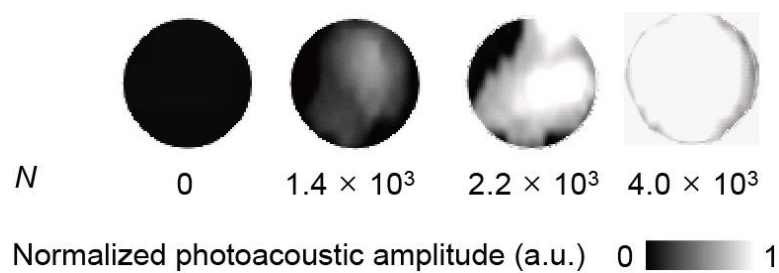
- [S1] Q. Zhang, W. Li, L.-P. Chen, and Y. Xia, *Chem. Eur. J.* **2010**, *16*, 10234–10239.
- [S2] K. H. Song and L. V. Wang, *J. Biomed. Opt.*, **2007**, *12*, 060503-(1–3).
- [S3] K. Maslov, G. Stoica, and L. V. Wang, *Opt. Lett.*, **2005**, *30*, 625–627.



**Figure S1.** a) A calibration curve for the extinction (*Ext.*) peak of the c(RGDyK)-labeled AuNCs as a function of the concentration (*C*) of AuNCs in the culture medium. The dashed line was a linear regression fit to the data ( $R^2=0.99$ ). b) Extinction (*Ext.*) spectra of the AuNCs dispersed in the culture medium before (solid line) and after incubation with the cells (dashed line). Extinction spectrum after the cellular uptake was normalized by considering the volume (2 mL) of the washing media (see experimental section in the Supporting Information). The initial concentration of the nanocages in the culture medium was 25 pM (based on particle number).



**Figure S2.** Superimposed two-photon and phase contrast images showing all the 14 mother-daughter cell groups containing the c(RGDyK)-labeled AuNCs. All the mother cells had roughly the same number of AuNCs, about  $4 \times 10^3$  per cell.



**Figure S3.** PAM images taken immediately after subcutaneous injection of U87-MG cells (20  $\mu$ L,  $1 \times 10^7$  cells/mL) containing different numbers of nanocages per cell: 0,  $1.4 \times 10^3$ ,  $2.2 \times 10^3$ , and  $4.0 \times 10^3$ , into a nude mouse.